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GENETICS IDENTIFICATION OF SEA HOLLY (*Acanthus ilicifolius*) THROUGH DNA BARCODING FROM COASTAL CILACAP, CENTRAL JAVA, INDONESIA**

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Running title: DNA barcoding sea holly in Indonesia

ABSTRACT

Information on molecular taxonomy of Sea Holly (*A. ilicifolius*) in coastal Cilacap, Central Java, Indonesia, was very limited. The present study aims to identified Sea Holly (*A. ilicifolius*) in coastal Cilacap. This study produced a reference library of molecular for Sea Holly (*A. ilicifolius*). The recently recorded species were utilized for the barcoding investigation. Genetic identification were evaluated by *rbcL* and *matK* gene. The young leaf samples of *A. ilicifolius* was extracted for DNA isolation and amplified with rbcL and matK primer. The length of rbcL gene was 608 bp, and the matK gene was 970 bp.The evolutionary history was build using the Neighbor-Joining Method. The barcodes sequences rbcL and matK analyzed with BLAST and MULTALIN. The sequences were also submitted to NCBI. From this study, we found that genera of *Acanthus (*Acanthaceae) and other genera were clustered in the same clade with high bootstrap value. The result indicated that locus of *rbcL* and *mat*K gene cannot be used for species differentiation in Acanthus, however, these genes can be used for distinguishing the genus level within Acanthaceae.

Keywords: *A. ilicifolius*, DNA barcode, matK, rbcL, sea holly

INTRODUCTION

Sea holly (*Acanthus ilicifolius)* are important mangrove plant. The plant commonly grows on the wetlands at the river mouth and coastal areas, and it is a true mangrove species (Ragavan *et al.* 2015). It is often distinguished from the related genera by its spiny leaves, spicate terminal inflorescences, two bracteoles and uniform anthers (Duke, 2006). In several countries, this plant functions as an efficacious medicianal plant (Simlai et al. 2013; Van et al. 2008; Kumar et al. 2008; Ganesh et al. 2008; Paul & Seenivasan 2017).

Identification based on characteristics of morphology, such as leaf shape, shape of flowers, branching stems and root shape is extremely prone to error (Sheue *et al*. 2009, Sahu and Kathiresan, 2012), as the morphology of *A. ilicifolius* cant be affected by geography.

Therefore, molecular identification is key in discriminating different species. In this study, we employed DNA barcoding, which uses short fragment of nucleotide sequence for fast, precise species identification (Dong *et al.* 2012; Kress *et al.* 2008; Li *et al.* 2012; Li *et al.* 2015; Vivas *et al.* 2014). DNA barcoding technology is still the ideal method for fast identification due to its convenience and low cost (Xu *et al* .2017).

Molecular identification using DNA barcoding is often needed to obtain fast, low cost and accurate identification. The DNA barcoding using mitochondrial DNA were employed to identity up to species level. The rbcl and matK genes from the chloroplast genome were used as the core barcode in the consortium for the Barcode of Life (CBOL Plant Working Group, 2009). The rbcL gene is the large subunit of Ribulose-bisphospate carboxlase gene. The matK gene is located with trnK gene and encodes the tRNA (Lys) (UUU). Substitution rate of the matK gene is highest among the plastid genes (Radulovici *et al.* 2010). DNA barcoding using matK can discriminate more than 90 % of species in the Orchidaceae but less than 49 % in the nutmeg family (Kress & Erickson 2007; Newmaster *et al.* 2008). In other research of the flora of Canada, genetic identification using rbcL and matK genes had revealed 93 % success in species identification, this methods can achieve discrimination up to 95 % with the addition of the trnH-psbA intergenic spacer (Burgess *et al.* 2011). In this study, we evaluated the efficiency of the matK and rbcL gene for the identification Sea Holly (*Acanthus ilifolicius*) from the coastal area of Cilacap.

MATERIALS AND METHODS

Collection of the sample

A total of 7 Young leaves individuals samples was collected from the west coast of Cilacap, with geographical latitude of 8°35' S - 8°48' S and longitude of 108°46' E - 109°03' E.

DNA Isolation

Total DNA were extracted from leaf tissue Sea Holly *(A. ilifolicius*) with Cetyl-trimethyl ammonium bromide (CTAB) (Sahu *et al.* 2016). The CTAB buffer was prepared from 20 mM EDTA; 1.4 M NaCl; 2 % PVP-30; 1 % β-mercaptoethanol; 10 % SDS and 10 mg/ml proteinase K and mixed with leaf sample. The suspension was incubated at 60 °C for 60 min and after this, suspension was centrifuged at 14,000 rpm for 10 min at room temperature with equal volume of CIAA (24:1). The aqueous phase was transferred to a new tube and precipitated with 0.6 volume of cold isopropanol (−20 °C) and chilled 7.5 M

ammonium acetate followed by storage at −20 °C for 1 h. The precipitated DNA was centrifuged at 14,000 rpm for 10 min at 4 °C followed by washing with 70 % ethanol. DNA was finally dissolved in TE buffer (10 mM Tris–HCl, 1 mM Na2EDTA, pH 8.0). The quantity DNA was evaluated using agarose gel electrophoresis and quality of total DNA was evaluated with nanodrop (Thermo Scientific, USA).

PCR and DNA sequencing

The DNA samples were amplified with rbcL and matK plastid primers *rbcLa_* f: 5'-ATGTCACCACAAACZAGAGACTAAAGC-3', rbcL724 -r : 5'-"GTAAAATCAAGT CCACCRCG"-3', *matK_390-f : 5'*- "CGATCTATTCATT CAATATTTC"-3' and matK_ 1326-*r: 5'*TCTAGCACACGAAAGTCGAAGT-3' (CBOL Plant Working Group, 2009). The total mixture was 50-µl containing 10–20 ng of template DNA, 200 µM of dNTPs, 0.1 µM primers and 1 unit of Taq DNA polymerase (Thermo Scientific, USA). The temperature profile of the PCR cycle for rbcL was 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; repeated for 35 cycles, and final extension 72 °C for 10 minute. For the amplification of the matK gene, the temperatures used were 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 50 °C for 40 s, 72 °C for 40 s; repeated for 37 cycles, and final extension72 °C for 5 minute. The amplified products were separated by agarose gel (1.2 %) electrophoresis and stained with ethidium bromide (Saddhe *et al.* 2016). PCR was conducted at Research Laboratory Jenderal Soedirman University, Indonesia and sequensing analysis were perform in 1st Base DNA sequensing service, Malaysia.

Phylogenetic analysis

A phylogenetic tree was constructed with http://www.phylogeny.fr for identified the genetic similarity and differences. Sampel of rbcL and matK genes was compared to sequences from 8 species, retrieved from NCBI GenBank. Relationship in rbcL and matK genes was generated with CLUSTAL W, Treeview version 1.5.2. was used to generated scoring method percent and the unrooted tree. The rbcL sample sequence compare with *Acanthus spinosus (*MF349678.1)*, Acanthus montanus* (L12592.1)*, Sclerochiton kirkii* (JX572958.1), *Acanthopsis spatularis* (KF724239.1), *Acanthus ilifolius* (KM255065.1), *Acanthus ilifolius* (KX231351.1), *Acanthus ebracteatus* (KX231352.1), *Acanthus ilifolius* (KP697342.1) (Figure 2). The matK sequence compare with *Acanthus ilifolius* (KX231339.1), *Acanthus ilifolius* (KM255080.1), *Acanthus ebracteatus* (KX231340.1), *Acanthus longifolius* (AJ429326.1), *Acanthus spinosus* (MF350143.1), *Acanthus mollis*

(HE967332.1), *Acanthus montanus* (HQ384511.1), *Aphelandra scabra* (JQ586377.1) (Figure 3).

Data analysis

Sequence alignment for rbcL and matK sequences was generated using Multalin V.5.4.1 (http://multalin.toulouse.inra.fr/multalin/). All known sea holly sequences were searched using the 'BLASTn' tool against the NCBI database and highest-scoring hit from each query is taken as the mangrove identification (https://www.ebi.ac.uk/services and https://www.ncbi.nlm.nih.gov). Similarity percentage matrix was calculated based on Clustal 2.1 (www.ebi.ac.uk). The rbcL sample sequence for similarity percentage compare with *Acanthus ilicifolius* (KP697342.1), *Acanthus ilicifolius* (KM255065.1), *Acanthus ilicifolius* (KX231351.1), *Acanthus ebracteatus* (KX231352.1), *Acanthopsis spathularis* (KF724239.1), *Acanthus montanus* (L12592.1), *Sclerochiton kirkii* (JX572958.1), *Acanthus spinosus* (MF349678.1) (Table 1) and the number of different nucleotides compare with *Acanthus ilicifolius* (KP697342.1), *Acanthus ebracteatus* (KX231352.1), *Acanthopsis spathularis* (KF724239.1), *Acanthus montanus* (L12592.1), *Sclerochiton kirkii* (JX572958.1), *Acanthus spinosus* (MF349678.1), *Aphelandra aurantiaca* (MF349506.1), *Aphelandra scabra* (JQ590024.1), *Sclerochiton harveyanus* (JX572957.1), *Crossandra infundibuliformis* (JQ933287.1), *Rhinacanthus nasutus* (KF381120.1), *Aphelandra sinclairana* (L01884.1), *Avicennia officinalis* (KP697352.1), *Avicennia marina* (KP697350.1), *Ruellia blechum* (GU135168.1), *Stachytarpheta jamaicensis* (JQ618493.1) (Table 2).

The Similarity percentage of matK sample sequence compare with *Acanthus ilicifolius* (KX231339.1), *Acanthus ebracteatus* (KX231340.1), *Acanthus ilicifolius* (KM255080.1), *Acanthus montanus* (HQ384511.1), *Acanthus spinosus* (MF350143.1), *Acanthus longifolius* (AJ429326.1), *Acanthus mollis* (HE967332.1), *Aphelandra scabra* (JQ586377.1) (Table 3) and the number of different nucleotides compare with *Acanthus ilicifolius* (KX231339.1), *Acanthus ebracteatus* (KX231340.1), *Acanthus ilicifolius* (KM255080.1), *Acanthus montanus* (HQ384511.1), *Acanthus spinosus* (MF350143.1), *Acanthus longifolius* (AJ429326.1), *Acanthus mollis* (HE967332.1), *Aphelandra scabra* (JQ586377.1), *Aphelandra aurantiaca* (JQ589891.1), *Aphelandra sinclairiana* (GQ981937.1), *Kudoacanthus albonervosus* (KX526470.1), *Sclerochiton harveyanus*

(JX517343.1), *Sclerochiton kirkii* (JX518192.1), *Proboscidea altheifolia (*MF963699.1) and *Schlegelia parviflora (*AJ429345.1) (Table 4).

RESULTS AND DISCUSSION

Identification of sea holly (*A. ilicifolius*) used rbcL and matK gene

Currently, morphology-based identification and the declining group of taxonomists result in weaks identification of species. This problem can be solved by using of molecular method for mangrove species identification. DNA barcoding is designed to provide accurate, and automated species identifications through the use of molecular species tags based on short, standard gene regions (Fazekas *et al.* 2008; Sadde *et al.* 2016). In this research, we found that the plastid core markers rbcL and matK cannot used as DNA barcoding for based assessment of sea holly from coastal Cilacap. We successfully amplified partial rbcL and matK gene from chloroplast genome of sea holly (Figure 1).

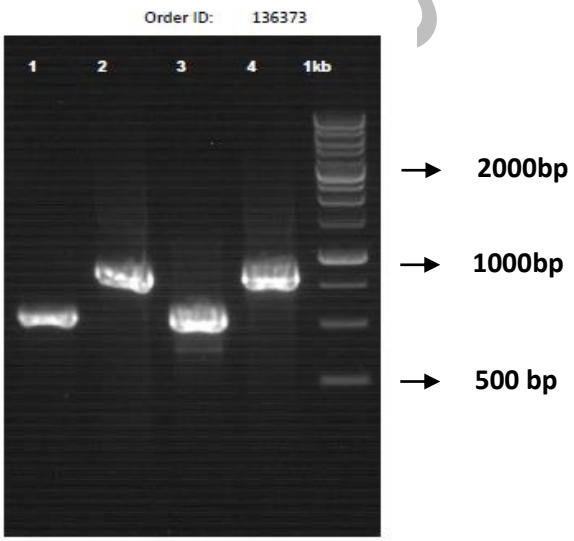

Figure 1. The Amplification of rbcL gene (608 bp) and matK gene (970 bp) of *Sea HolLy (A. ilicifolius)* and used 1 kb marker.

Table 1. Similarity percentage of Sea Holly (*A. ilicifolius)* base on rbcL gene with *Acanthus ebracteatus*, *Acanthopsis spathularis*, *Acanthus montanus*, *Sclerochiton kirkii* and *Acanthus spinosus* calculated using Clustal 2.1 (www.ebi.ac.uk)

Species	1	2	3	4	5	6	7
KP697342.1_*Acanthus ilicifolius*	100	100	100	100	100	100	100
KM255065.1_ *Acanthus ilicifolius*	100	100	100	100	100	100	100
KX231351.1_*Acanthus ilicifolius*	100	100	100	100	100	100	100
KX231352.1_*Acanthus ebracteatus*	100	99.29	98.3	98.3	98.3	98.3	98.4
KF724239.1_*Acanthopsis spathularis*	98.4	98.1	98.1	98.1	98.1	98.1	98
L12592.1_ *Acanthus montanus*	98.2	98.2	98.2	98.2	98.2	98.2	98.3

JX572958.1_*Sclerochiton kirkii*	98.2	98.2	98.2	98.2	98.2	98.2	98.2
MF349678.1_*Acanthus spinosus*	98.0	98.4	98.0	98.0	98.0	98.0	98.0

The DNA sequence of *rbcL* and *matK* gene was perform in BLAST at NCBI to find similar sequence under the same or different genus within family Acanthaceae and calculated using Clustal 2.1 (www.ebi.ac.uk). The most highly similar identity sequences obtained from the GenBank base on rbcL gene are *Acanthus ilicifolius* (100%), *Acanthus ebracteatus* (100%), *Acanthopsis spathularis* (98.4%), *Acanthus montanus* (98.2%), *Sclerochiton kirkii* (98.2%), *Acanthus spinosus* (98.0%) (Table 1). The similarity was 98.4% with *Acanthopsis spathularis*, which has 9 nucleotides difference with all *A. ilicifolius*. *Acanthus montanus* has 10 nucleotides difference with all *A. ilicifolius* with 98.02% similarity (Table 2).

Table 2. Number of different nucleotides in *rbcL* gene of Sea Holly (*A. ilicifolius*) and other species within Acanthaceae.

Species	Accession number	Identity (%)	Number of different nucleotides
Acanthus ilicifolius	Sea holly	100	0
Acanthus ebracteatus	KX231352.1	100	0
Acanthopsis spathularis	KF724239.1	98.4	9
Acanthus montanus	L12592.1	98.2	10
Sclerochiton kirkii	JX572958.1	98.2	10
Acanthus spinosus	MF349678.1	98.0	11
Aphelandra aurantiaca	MF349506.1	98.0	11
Aphelandra scabra	JQ590024.1	98.0	11
Sclerochiton harveyanus	JX572957.1	98.0	11
Crossandra infundibuliformis	JQ933287.1	98.0	11
Rhinacanthus nasutus	KF381120.1	97.4	14
Aphelandra sinclairana	L01884.1	97.7	17
Avicennia officinalis	KP697352.1	96.4	20
Avicennia marina	KP697350.1	96.4	20
Ruellia blechum	GU135168.1	95.0	22
Stachytarpheta jamaicensis	JQ618493.1	95.0	22

Table 3. Similarity percentage and of Sea Holly (*A. ilicifolius)* based on matK gene with *Acanthus ebracteatus*, *Acanthopsis spathularis*, *Rhinacanthus nasutus* and *Acanthus spinosus*

Species	1	2	3	4	5	6	7
KX231339.1_*Acanthus ilicifolius*	99.3	99.3	99.3	99.3	99.3	99.3	99.3
KX231340.1_*Acanthus ebracteatus*	99.3	99.3	99.3	99.3	99.3	99.3	99.3
KM255080.1_ *Acanthus ilicifolius*	97.9	97.9	97.9	97.9	97.9	97.9	97.9
HQ384511.1_*Acanthus montanus*	96.5	96.5	96.5	96.5	96.5	96.5	96.5
MF350143.1_*Acanthus spinosus*	96.5	96.5	96.5	96.5	96.5	96.5	96.5
AJ429326.1_*Acanthus longifolius*	96.4	96.4	96.4	96.4	96.4	96.4	96.4

HE967332.1_*Acanthus mollis*	96.3	96.3	96.3	96.3	96.3	96.3	96.3
JQ586377.1_*Aphelandra scabra*	94.8	94.8	94.8	94.8	94.8	94.8	94.8

Table 4. Number of different nucleotides in *matK* gene of Sea Holly (*A. ilicifolius*) and other species within Acanthaceae retrieved from GenBank

Species	Accession number	Identity (%)	Number of different nucleotides
Acanthus ilicifolius	KX231339.1	99.3	6
Acanthus ebracteatus	KX231340.1	99.3	6
Acanthus ilicifolius	KM255080.1	98.1	8
Acanthus montanus	HQ384511.1	96.5	19
Acanthus spinosus	MF350143.1	96.5	26
Acanthus longifolius	AJ429326.1	96.4	23
Acanthus mollis	HE967332.1	96.3	27
Aphelandra scabra	JQ586377.1	94.8	40
Aphelandra aurantiaca	JQ589891.1	94.5	41
Aphelandra sinclairiana	GQ981937.1	94.5	42
Kudoacanthus albonervosus	KX526470.1	94.4	47
Sclerochiton harveyanus	JX517343.1	93.8	48
Sclerochiton kirkii	JX518192.1	93.7	49
Proboscidea altheifolia	MF963699.1	91.4	75
Schlegelia parviflora	AJ429345.1	91.3	79

The *MatK* gene showed poor performance at species level identification, Sea Holly has 99.3% similarity with *Acanthus ebracteatus* (Table 3 and Table 4). Species differentiation was relatively weak for matK in the research of DNA barcoding of Poaceae (Saadullah *et al.* 2016). Single locus of DNA barcode could not provide an significant level of differentiation. Therefore a combination of 2 loci with 4 available markers is needed to determine the ability to species differentiation (Gonzalez *et al.* 2009). Combination of rbcL + matK gene for species differentiation in the reseach of tropical tree species in Guiana was lower (<50 %) (Gonzalez et al. 2009). Lower discrimination was reported in complex and closest taxa of *Holcoglossum, Lysimachia*, *Curcuma* and *Ficus* using matK and rbcL gene (Xiang *et al.* 2011; Zhang *et al.* 2012; Li *et al.* 2012; Chen *et al.* 2015). Differentiation with 2-loci *rbc*L + *trn*H-*psb*A and *mat*K + ITS had result 100% differences between species (Purushosthaman *et al*. 2014). The combination between *rbcL* + *matK* markers results better performance at species and genus level identification (Sadde *et al.* 2016). Another study report only 72 % is species level resolution using combined between *matK* and *rbcL* (Saddhe *et al.* 2017).

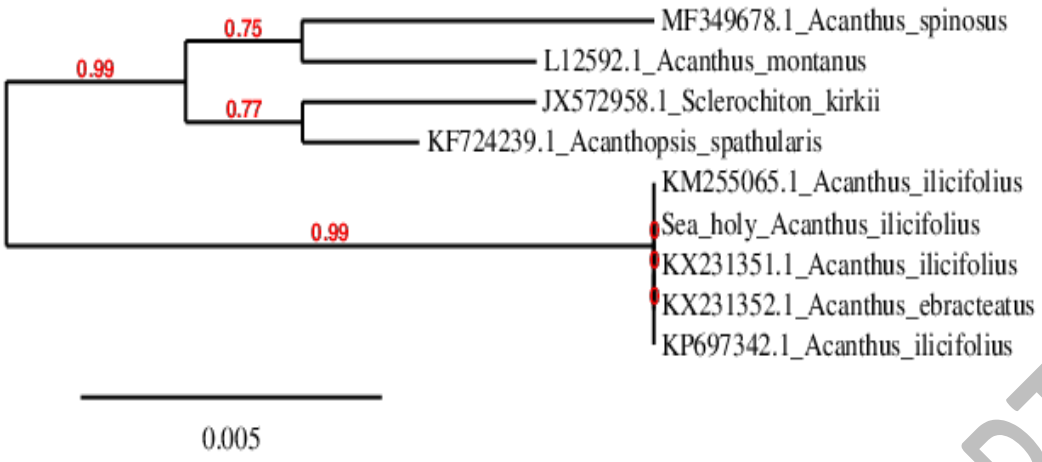

Figure 2. Phylogenetic tree of genus *Acanthus*, *Acanthopsis*, and *Sclerochiton* constructed based on likelihood phylogeny of nucleotide sequences of *rbcL* gene.

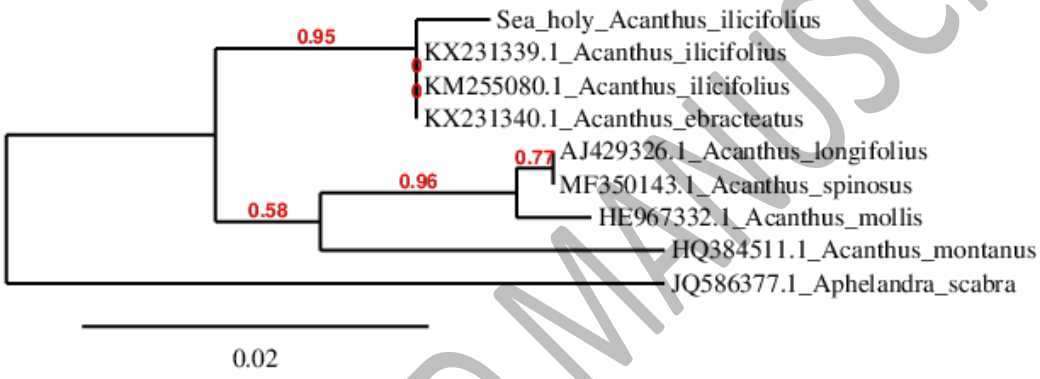

Figure 3. Phylogenetic tree of genus *Acanthus* and *aplelandra* constructed based on likelihood phylogeny of nucleotide sequences of matK gene.

The results showed that *rbcL* locus could not differentiated Sea Holly from *Acanthus ilicifolius* and *Acanthus ebrecteatus*, but can differentiate with *Acanthus montanus*, *Acanthus spinosus* and others genera (*Acanthopsis*, and *Sclerochiton*) (Figure 2). The result from phylogenetic tree also showed that *matK* locus could not differentiate Sea Holly from *Acanthus ebrecteatus*, otherwise *matK* locus can separate *Acanthus longifolius, Acanthus spinosus, Acanthus mollis, acanthus montanus*, and *Aphelandra scabra* (Figure 3). The Phylogenetic tree was build from the highest likelihood in sequence alignment. The number of genetic change build from horizontal dimension of the phylogenetic tree. Value of 0.005 and 0.002 explains the length of the branch representing the number of nucleotide substitution (number of substitution per 100 nucleotide site). There was no indel (insertion and deletion) found in *rbcL* and *matK* gene sequence of Sea Holly (*A. ilicifolius).*

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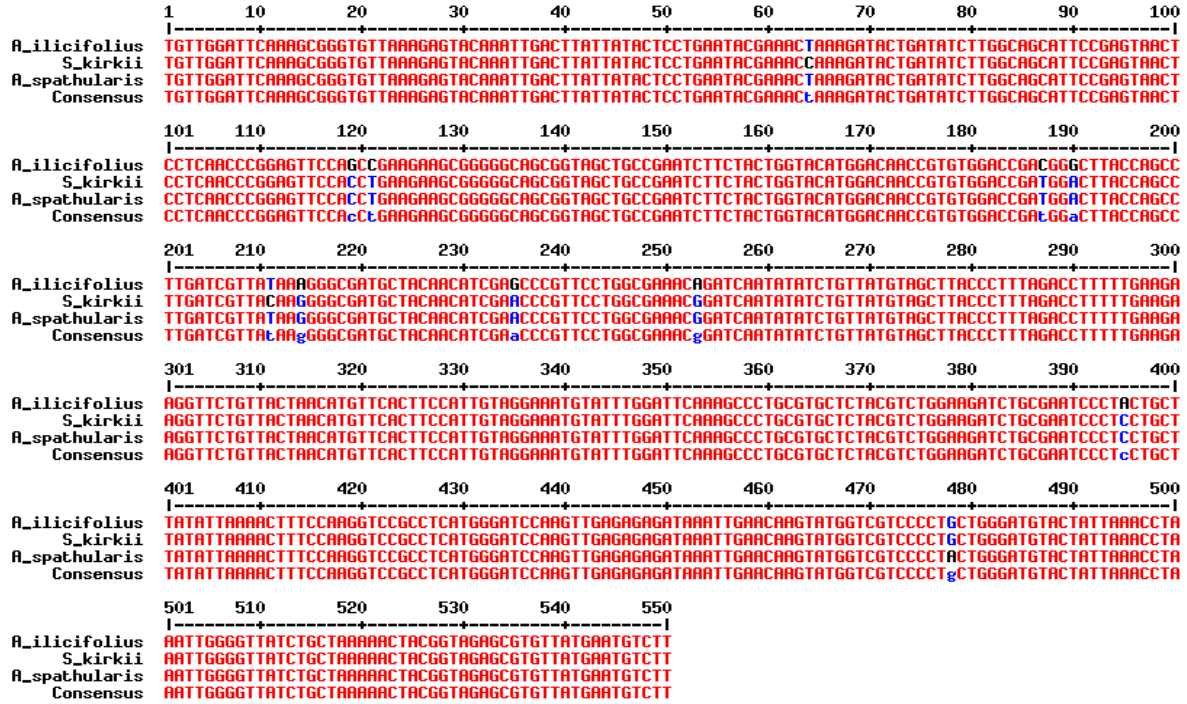

Figure 4. Multi Alignment of *rbcL* gene sequence of *Acanthus ilicifolius*, *Acanthopsis spathularis*, and *Sclerochiton kirkii*

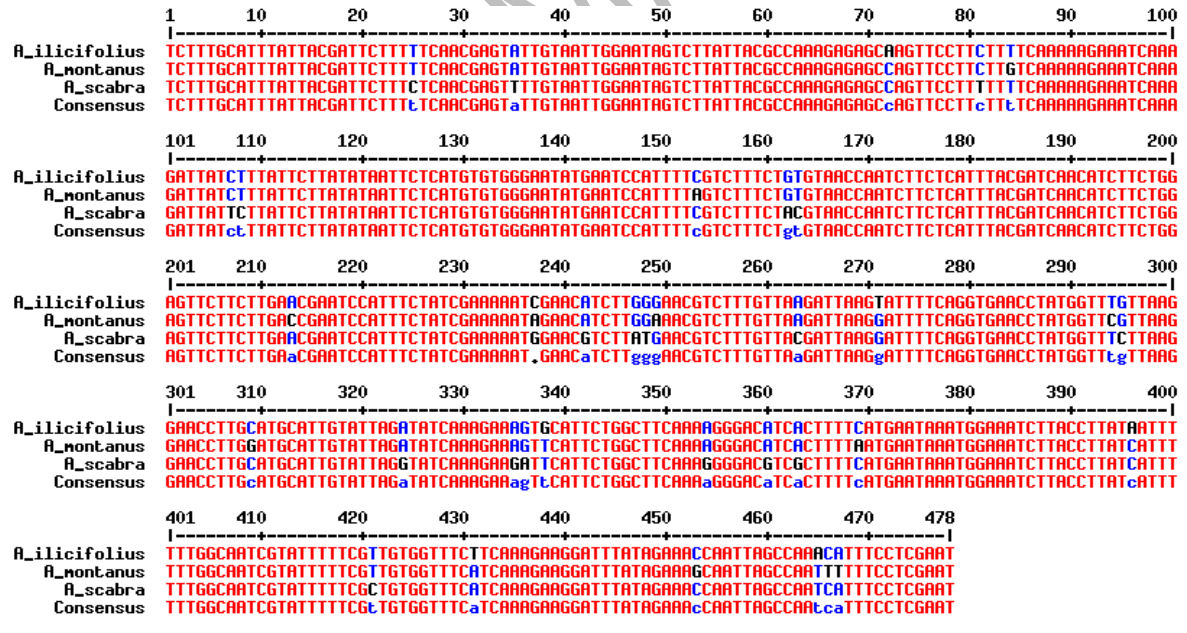

Figure 5. Multi Alignment of *matK* gene sequence of *Acanthus ilicifolius*, *Acanthus montanus* and *Aphelandra scabra*.

The position of different nucleotides in species with the same or a different number of nucleotide difference was not the same in rbcL and matK. As seen in alignment result amongst *Acanthus ilicifolius*, *Acanthopsis spathularis*, and *Sclerochiton kirkii* (Figure 4) has

11 point of different nucleotide and *Acanthus ilicifolius, Acanthus montanus* and *Aphelandra scabra* (Figure 5) has 36 points of different nucleotide. The result of multi alignment of *rbcL* sequences has been little variation compared with multi alignment of *matK* sequences. These results are in line with Saddhe *et al* 2016 and Harisam *et al* 2018, that matK gene more sensitive compared with rbcL gene.

There are many molecular marker techniques that are used to identify tropical mangrove species. Several studies suggested that one marker alone was not accurate enough for identification (Dong *et al*. 2012). The *rbcL* and *mat*K gene can be considered as a barcode for mangrove species. The highest rate of sequencing was observed in *rbcL* (98.7 %), while amplification as well as sequencing rate of *matK*, was 99 %.

DNA barcode was primary essential for identification in mangrove. Genetic diversity assessment is also essential in endemic species, endangered and rarely found. This information was important for mangrove conservation. Species identification in this research was performed using *rbcL* and *matK* sequence. From this results showed that *rbcL* and *matK* sequence genes cannot separate *A. ilicifolius* from *A. ebracteatus*. However another research in terestrial plants were success identified with rbcl and matk sequence (Hollingsworth *et al.* 2009; Kress *et al.* 2010; Kuzmina *et al.* 2012; Sadde *et al.* 2016). The species were assigned to their taxa based on two methods, the similarity-based method using BLAST score based single linkage (BLASTClust) and tree-based method (NJ). Sadde *et al.* (2016) report that the combine of *rbcL* and *mat*K gene has significant variation and can be used for identification of *Acanthus ilicifolius* and other mangrove species in India. However, considering sea holly has 100% similarity with two other mentioned species, it is suggested that the *rbcL* gene cannot be used to differentiate *Acanthus* species (Table 2). In contrast, Ross and CBOL reported that species identification success rate using *rbcL* seems to be higher, whereas *rbcL* recovery ranged from 90 to 100 % (Ross *et al.* 2008; CBOL Plant Working Group 2009). While 9 nucleotides difference in *rbcL* gene can already place *Acanthopsis spathularis* as different species from *Acanthus ilicifolius*. Ideally, the range of minimum discrimination for discrimination species from 0.0 - 0.27%, or similarity of 99.74 - 100% to place organisms in the same species (Purushothaman *et al*. 2014). Sadde *et al.* (2016) report that *mat*K has significant variation and can be used for DNA barcode in acanthus family. The present coding regions of *mat*K and *rbc*L, which are often recommended in the DNA barcode research only show moderate variability in *Populus* genus and inefficient for use in species differentiation. In this result we can suggest that one marker alone such as *matK* and *rbcL*

gene were not helpful to identification mangroves at the species level Sea Holly (*Acanthus ilicifolius).*

CONCLUSION

Identification of Sea Holly (*Acanthus ilicifolius)* base on *mat*K and *rbc*L gene was not successful to differentiate at the species level, only successfully on the genus level**.** Single locus of DNA barcode could not provide an adequate level of differentiating, therefore the combination of more than two loci is suggested.

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